ON THE MECHANISM OF OXIDASE ACTION

G. B. Reed

(WITH FOUR FIGURES)

The suggestion was made some years ago by BACH,[1] and later by KASTLE and LOEVENHART,[2] that the action of the oxidases depends on the fact that these bodies have a special aptitude for forming peroxides; that molecular oxygen in contact with complex autoxidizable substances combines with them to form unstable peroxides which in turn can give up their oxygen, in part or completely, to any oxidizable substance present in the cell. These changes may be represented diagrammatically as follows: (1) $A + O_2 = AO_2$ (oxygenase); (2) $AO_2 + 2B = A + 2BO$; or (3) $AO_2 + B = AO + BO$; and (4) $AO_2 + A = 2AO$; where A is the autoxidizable substance (or substances) contained in the cell, which, by uniting with oxygen, forms the oxidase AO_2. The oxidase, in contact with an oxidizable substance B, may give up its oxygen in a variety of ways, depending upon conditions. All of the oxygen may be absorbed by B as in equation (2), in which case A is liberated and may begin the cycle again by combining with atmospheric oxygen; but if only a part of the oxygen of the oxidase is absorbed, as in equation (3), the resulting AO is assumed to be inert and incapable of regenerating new oxygenase. Finally, if no combustible substances are present, a part of the oxygen of the oxygenase may combine with a second A, which thus loses its regenerative power.

This conception of biological oxidation, originally based upon TRAUBE's peroxide theory of oxidation, finds abundant support in many discoveries made during the last 10 years. TRAUBE was of the opinion that hydrogen peroxide is formed as a primary product in many, if not in all, oxidation processes, and that through its agency oxygen is transferred to the bodies finally undergoing combustion. ENGLER and his collaborators have been able to show

[1] Compt. Rend. Acad. Sci. **124**:951–954. 1897.

[2] Amer. Chem. Jour. **26**:539–566. 1901.

that a peroxide of the substance undergoing oxidation is the usual intermediate product. Thus, when rubidium burns in air it is converted quantitatively into rubidium peroxide: $Rb+O_2=RbO_2$; and as BAEYER and VILLIGER[3] have shown, when benzaldehyde is exposed to oxygen, or to the air, it first absorbs oxygen to form benzoyl-hydrogen peroxide:

$$C_6H_5CHO+O_2=C_6H_5CO-O-OH.$$
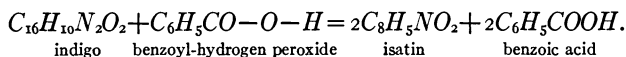benzaldehyde   benzoyl-hydrogen peroxide
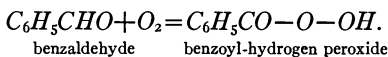
If allowed to remain in contact with water, the benzoyl-hydrogen peroxide is hydrolyzed with the production of benzoic acid and hydrogen peroxide:

$$C_6H_5CO-O-OH+HOH=C_6H_5COOH+H_2O_2.$$
benzoyl-hydrogen peroxide   benzoic acid
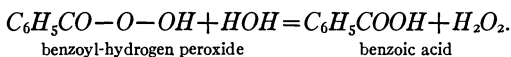
If a second molecule of benzaldehyde comes in contact with the benzoyl-hydrogen peroxide, however, the former is oxidized and two molecules of the acid are produced:

$$C_6H_5CO-O-OH+C_6H_5CHO=2C_6H_5COOH.$$
benzoyl-hydrogen peroxide   benzaldehyde   benzoic acid
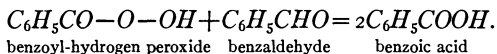
Finally, if an oxidizable substance, such as indigo, be present, it is oxidized by the benzoyl-hydrogen peroxide with the production of isatin and benzoic acid, thus:

$$C_{16}H_{10}N_2O_2+C_6H_5CO-O-H=2C_8H_5NO_2+2C_6H_5COOH.$$
indigo   benzoyl-hydrogen peroxide   isatin   benzoic acid

Hence through the intermediary action of the benzoyl-hydrogen peroxide benzaldehyde is oxidized to benzoic acid; or a second oxidizable substance, which is not acted upon by atmospheric oxygen, may also be oxidized by the peroxide.

More direct evidence that the oxidations within the organism follow this peroxidase procedure has been given by BACH and CHODAT,[4] who were able to isolate a peroxide from the fresh juice of *Lathraea squamaria* at a time when it exhibited active oxidase action. After long standing, however, when the juice had lost its oxidase activity, it contained no peroxide. These investigators accordingly concluded that the oxidase itself is of a peroxide nature or contains a peroxide as one of its constituents. This conclusion

[3] Ber. Deutsch. Chem. Gesells. **33**:1569–1585. 1900.

[4] Ber. Deutsch. Chem. Gesells. **35**:2466–2470. 1902.

was later[5] confirmed and extended by separating an oxidase into two constituents, a peroxide-like substance which they called oxygenase, and a body capable of activating this peroxide, namely, a peroxidase. By treating the fresh juice of *Lactarius vellereus* with 40 per cent alcohol, a precipitate was obtained which (in watery solutions) exhibited the properties of a very weak oxidase. The filtrate, however, when taken alone showed no oxidative activity, but was capable of imparting great activity to either hydrogen peroxide or to the precipitate which contained the oxygenase.

A considerable number of data indicate that all the so-called "direct oxidases" (that is, those which blue gum guaiac without the addition of hydrogen peroxide) consist of two such substances. BACH[6] has shown that the peroxidase separated from tyrosinase will activate either its own oxygenase or hydrogen peroxide. The writer has also obtained similar results with an oxidase of the laccase type from potatoes by the following method.

Equal volumes of finely grated potato peelings and 55 per cent alcohol were mixed in a mortar, ground together until the potato was completely macerated, and then filtered. The filtrate was found to contain a peroxidase; while the residue, after washing with 55 per cent alcohol, extracting for several hours with an equal volume of water and finally filtering, gave an oxygenase solution, as the following experiments indicate.

The oxidizing activity of each of these fractions on pyrogallol, acting separately or in conjunction, was determined by mixing them in the proportions indicated in table I. After 4 hours the

TABLE I

cc. 5 per cent pyrogallol	cc.potato ·oxygenase	cc.potato peroxidase	cc. horse-radish peroxidase	cc.water	cc.0.05M KMnO$_4$ required for titration
25.	10	. . . .	. . . .	10	1.4
25.	. . . .	10	. . . .	10	0.3
25. . . . .·.	. . . .	. . . .	10	10	0.8
25.	10	10	. . . .	. . . .	8.2
25.	10	. . . .	10	. . . .	6.4

[5] Ber. Deutsch. Chem. Gesells. **36**:600–605. 1903.

[6] Ber. Deutsch. Chem. Gesells. **39**:2126–2129, 3329–3331. 1906.

purpurogallin resulting from the oxidation of the pyrogallol was filtered off on asbestos under pressure, washed free of unoxidized pyrogallol, dissolved in 10 cc. of concentrated sulphuric acid, and the amount determined by titrating with 0.05M potassium permanganate. The results are indicated in the last column of table I.

The amount of pyrogallol oxidized in 4 hours by different combinations of potato and horse-radish peroxidase with potato oxygenase is indicated by the amount of $KMnO_4$ required for titration (the greater the amount of $KMnO_4$ required the greater the oxidation). It is evident that, like the *Lactarius* oxygenase, the potato oxygenase has some power to oxidize pyrogallol, but that this action is greatly accelerated by the peroxidase from the same source and to a less extent by that obtained from other sources (for example, from the horse-radish).

These results furnish additional evidence in support of the conclusion that all the *direct* oxidases consist of oxygenases plus peroxidases. Some tissues or extracts possess the *indirect* oxidase action (that is, they react only after the addition of a peroxide) and are capable of activating the oxygenases, hydrogen peroxide, or various organic peroxides, and it appears that they differ from those exhibiting *direct* action only in the absence of oxygenase or in lacking the ability to regenerate oxygenase.

BACH and CHODAT[7] consider that the oxygenases are formed as a result of an enzyme reaction or that they are themselves enzymes. MOORE and WHITLEY,[8] while agreeing with the former investigators as to the occurrence of oxygenases, consider them to be merely unstable peroxides resulting directly from contact of atmospheric oxygen with various substances in the cell, such as BAEYER and VILLIGER (*l.c.*) have shown that benzoyl-hydrogen peroxide will form. The solution of this problem awaits further investigation.

Whatever the origin of the oxygenase may be, it is clear that there is a substance, peroxidase, possessing enzyme properties, and capable of transferring oxygen from oxygenases (or from peroxides of known constitution) to oxidizable substances. The

---

[7] Ber. Deutsch. Chem. Gesells. **36**:600–605. 1903.

[8] Biochem. Jour. **4**:136–167. 1909.

mechanism of this reaction, however, has been quite unknown. A suggestion in regard to it was made by BACH and CHODAT,[9] who found that an excess of either peroxidase or hydrogen peroxide had no effect on the rate at which pyrogallol was oxidized by hydrogen peroxides and horse-radish peroxidase. They concluded that the peroxide and peroxidase always take part in the reaction in constant proportions. BACH and CHODAT thus arrived at the conclusion which had previously been advanced by KASTLE and LOEVENHART (*l.c.*) from theoretical considerations, namely, that the peroxidase forms a definite compound with hydrogen peroxide, exhibiting more energetic oxidizing properties than the peroxide alone.

The writer has obtained results of a much more definite and conclusive character by experimenting with platinum black and by applying the suggestions gained in this way to the study of enzyme reactions found in living tissue. The present paper contains an account of the experiments with platinum black.

In connection with certain experiments it was observed that different samples of colloidal platinum and colloidal silver (prepared by passing a direct current between two electrodes of the metal under water) behaved differently toward solutions of gum guaiac. In some cases the guaiac was oxidized directly, in others only after the addition of a peroxide. This condition suggested to the writer that samples of colloidal metal might contain different proportions of oxygen, and, moreover, that the amount might be varied by suitable treatment. Subsequent experimentation has proved this supposition to be correct.

Instead of a solution of colloidal platinum, a platinum surface covered with the colloidal metal has been employed. A large platinum crucible with a surface of about 150 sq. cm. was platinized in the ordinary manner, by making it a cathode in a solution containing 2 gm. platinum chloride and 0.16 gm. lead acetate in 60 cc. of water, until its surface was uniformly coated with a black deposit of the colloidal metal. It was then subjected to active hydrogen by making it a cathode in a dilute solution of hydrochloric acid through which was passed a current of one ampere at 110 volt. To prevent the oxygen generated at the opposite pole from reaching

9 Ber. Deutsch. Chem. Gesells. **37**:1342–1348. 1904.

the colloidal platinum the electrolysis was carried out in two beakers connected by a siphon.
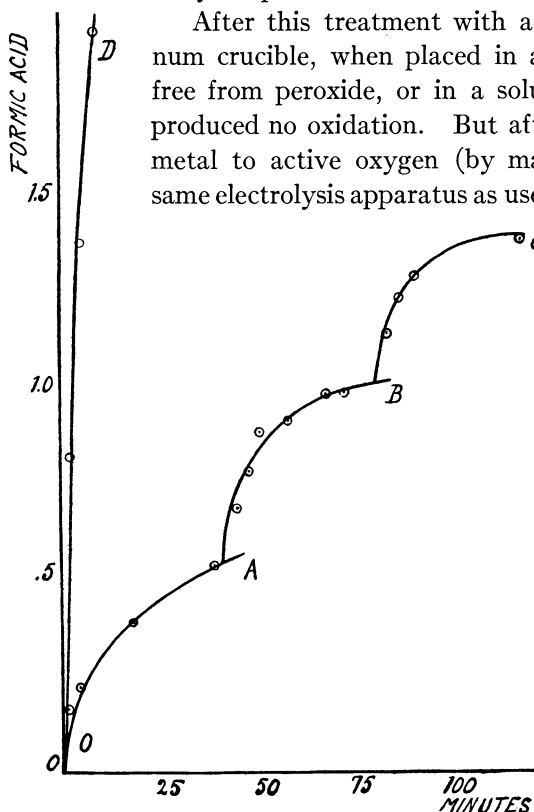
After this treatment with active hydrogen, the platinum crucible, when placed in a solution of gum guaiac[10] free from peroxide, or in a solution of potassium iodide, produced no oxidation. But after subjecting the colloidal metal to active oxygen (by making it the anode in the same electrolysis apparatus as used before) it produced rapid oxidation when placed in a solution of either gum guaiac or potassium iodide. This was shown by the appearance of a blue color in the former and after the addition of starch paste in the latter. Although this reaction took place rapidly, it was observed that only a very small amount of material was oxidized. This suggested that the amount of oxygen taken up by the platinum was sufficient for only a limited oxidation.

To determine whether this was true, quantitative experiments were undertaken. For this purpose the oxidation of formic aldehyde to formic acid suggested itself as a reaction, which is

FIG. 1.—Curves showing oxidation of formaldehyde; lower curves OA, AB, BC represent successive oxidations by platinum black which had been charged with oxygen by making it an anode in dilute acid at the beginning of each operation; upper curve OD represents the oxidation by hydrogen peroxide in the presence of platinum black; ordinates represent amount of formic acid produced (expressed as the difference in the number of cc. 0.05M HCl required to neutralize the NaOH in 5 cc. of the mixture at the beginning of the experiment and after a part of it had been neutralized by the formic acid produced); abscissae represent time in minutes.

[10] It was shown by MOORE and WHITLEY (Biochem. Jour. 4:169) that ordinary alcoholic solutions of gum guaiac frequently contain traces of peroxide which may be removed by boiling the tincture with animal charcoal.

catalyzed by platinum black and which can be accurately and conveniently measured.

About 80 cc. of a solution containing 0.05M NaOH and approximately 0.3M formaldehyde was placed in an open beaker in a water bath maintained at a constant temperature of 30° C. The platinum crucible was freshly platinized and exposed to active oxygen (as previously described) for 5 minutes. It was then washed by rapidly dipping it into three changes of distilled water,[11] after which it was introduced into the solution of formaldehyde. The mixture was kept thoroughly stirred throughout the experiment, and at frequent intervals 2 cc. were removed and titrated with 0.05M HCl. This showed the amount of NaOH neutralized by the formic acid generated in the reaction. The results are expressed in the curve OA, fig. 1.

The amount of formic acid formed in this reaction is very small when compared with the amount of formic aldehyde present at the start. When equilibrium is reached, therefore, it must be the oxygen (presumably the oxygen that was combined with the platinum in the charging process), and not the aldehyde, that is used up. If this be true, by furnishing more oxygen combined with the platinum the reaction should proceed much farther. This condition was realized experimentally by recharging the platinum with oxygen.

After the first reaction between oxidized platinum and aldehyde had almost reached an equilibrium, the platinum electrode was removed from the solution and subjected to active oxygen as in the former case; it was then washed and returned to the same formaldehyde solution. Renewing the supply of oxygen on the platinum in this manner caused the formation of more formic acid. The results plotted in the curve AB, fig. 1, indicate that the reaction proceeded at approximately the same rate as with the first charge of oxygen. Repeating the operation a third time gave the similar results plotted in the curve BC, fig. 1.

[11] As a check experiment, the platinum after this amount of washing was placed in 0.05M NaOH without formaldehyde; titration showed no decrease in alkalinity, hence the washing was sufficient to remove the very dilute acid of the solution in which it was oxidized.

It is evident that the oxygen concerned in this reaction is derived from the platinum, and that the more frequently the platinum is recharged with oxygen the higher will be the rate of formation of formic acid. It is of theoretical interest to determine whether the rate can thus be made to approximate that of the production of formic acid in the presence of hydrogen peroxide. The latter reaction was accordingly investigated.

Since hydrogen peroxide has considerable oxidizing action on the aldehyde in the absence of a catalyzer, it was necessary first of all to determine the effect of the colloidal platinum on the reaction. For this purpose the following method was adopted. To a solution containing the same concentration of formic aldehyde and sodium hydroxide as used in the previous experiment sufficient hydrogen peroxide was added to make the concentration 0.5M. The rate of oxidation of the
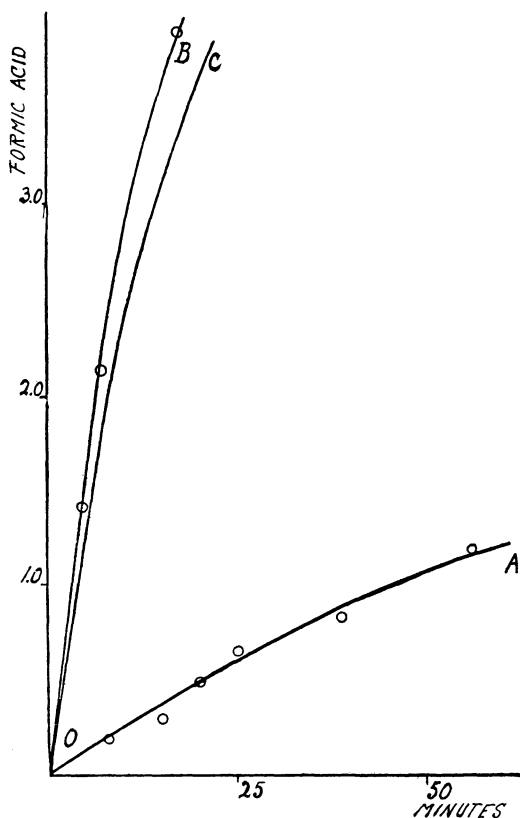
Fig. 2.—Curves of the rate of oxidation of formaldehyde by hydrogen peroxide: *OA* represents oxidation in absence of a catalyzer; *OB* represents oxidation in presence of colloidal platinum; *OC* represents activity of the catalyzer; ordinates represent amount of formic acid produced (stated as the difference in number of cc. 0.05M HCl required to neutralize the NaOH in 5 cc. of the mixture at the beginning of the experiment and after a part of it was neutralized by the formic acid produced); abscissae represent time in minutes.

formic aldehyde in the mixture (which was kept well stirred and at a constant temperature of 30° C.) was determined, as in the former

case, by titrating samples at frequent intervals with HCl. The results are indicated by the curve *OA*, fig. 2. The freshly platinized platinum crucible was then placed in a similar mixture of formic aldehyde, alkali, and hydrogen peroxide, and the rate of oxidation under the new conditions determined as before. The results are shown in the curve *OB*, fig. 2. By subtracting the ordinates of the curve *OA* from the ordinates of *OB* we obtain the curve *OC*, which expresses the effect of the catalyzer. For subsequent comparison the curve *OC* is also plotted as curve *OD*, fig. 1.

As has been shown, the speed of the reaction between aldehyde and oxidized platinum, in the absence of any other source of oxygen, is a function of the amount of oxygen which can be furnished by the charged platinum; and it is evident from an inspection of the reaction curve *OA* of fig. 1 that by starting with a sufficiently high concentration of oxygen on the platinum the initial velocity could be made equal to the velocity of the catalyzed peroxide reaction represented by *OD*. As the curves indicate, the former reaction in the absence of a continual supply of oxygen slows down much more rapidly than the peroxide reaction; but it is clearly possible to prevent this by recharging the platinum with oxygen at sufficiently short intervals. It seems evident, therefore, that the hydrogen peroxide acts by recharging the platinum with oxygen as soon as a portion of the oxygen has been removed by the formaldehyde. This is of great theoretical interest as an explanation of the means by which the catalyzer produces its effect, that is, by combining with one or both of the reacting substances.

Measurement of the oxidation potential of this platinized surface when connected as a cathode through a formic aldehyde mixture similar to that previously used led to the same conclusions.[12] When freshly charged with oxygen the platinum exhibited a *high* potential, but on contact with the aldehyde solution this rapidly dropped to equilibrium at very nearly zero potential. In the presence of hydrogen peroxide and the aldehyde mixture,

[12] REED, G. B., Measurement of oxidation potential and its significance in the study of oxidases. BOT. GAZ. 61:523–527. *figs. 2.* 1916.

however, the oxidation potential did not fall so low, and it remained approximately constant until the peroxide was exhausted.[13]

From these results it may be concluded that in the oxidation of formic aldehyde by hydrogen peroxide in the presence of platinum black two reactions are concerned: the platinum combines with oxygen from the hydrogen peroxide, as it combined with oxygen when subjected to anodic oxidation; this compound of platinum then gives up its oxygen to the formaldehyde, producing formic acid.

In a similar manner it was possible to show that the catalytic action of platinum black on the oxidation of potassium iodide by hydrogen peroxide was due to an intermediate platinum-oxygen compound. A freshly platinized platinum crucible, which had just been subjected to active oxygen in the manner described in the previous experiment, was
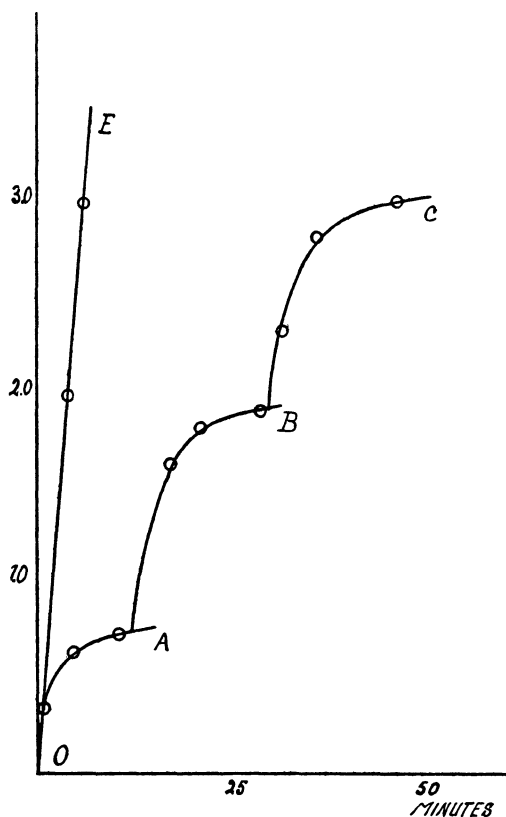
FIG. 3.—Curves showing oxidation of potassium iodide; lower curves *OA*, *AB*, *BC*, represent successive oxidations by platinum black which had been charged with oxygen at the beginning of each oxidation; curve *OE* represents oxidation by hydrogen peroxide in presence of colloidal platinum; ordinates represent drops of $0.01\,M\ Na_2\,S_2\,O_3$ required to combine with the iodine in 5 cc. of the reaction mixture; abscissae represent time in minutes.

[13] The oxidation potential in hydrogen peroxide is much lower than would be expected from its activity as an oxidizing agent (LEWIS, Jour. Amer. Chem. Soc. **36**: 1696), which is probably due to the fact that it may act as both an oxidizing and as a reducing agent.

placed in 100 cc. of a 0.2M solution of potassium iodide, which was maintained at a constant temperature of 20° C. Portions of 5 cc. were removed at frequent intervals and titrated with 0.01M sodium thiosulfate (with starch indicator) for the amount of iodine liberated in the oxidation. The curves *OA*, *AB*, *BC*, fig. 3, represent the velocity of oxidation of potassium iodide after successive chargings of the platinum with oxygen.
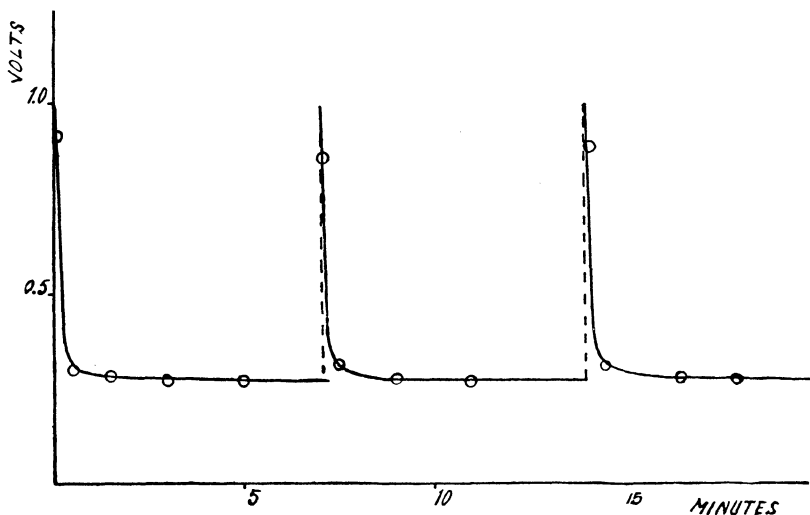

FIG. 4.—Curves showing fall of oxidation potential of colloidal platinum in 3 successive oxidations of a potassium iodide solution after the platinum had been charged with oxygen at an anode at the beginning of each oxidation; ordinates represent volts; abscissae represent time in minutes.

By connecting the platinum, as a cathode, to an apparatus for the measurement of the oxidation potential it was found that the rate at which the oxygen potential dropped, that is, the rate at which oxygen was given up by the platinum electrode in the potassium iodide solution, followed very nearly the velocity of the oxidation reaction. This may be seen by comparing the curves in fig. 4, showing the changes in the potential of the platinum after three successive chargings with oxygen, with the rates of oxidation produced by the platinum as plotted in fig. 3. It is evident that the drop in potential is somewhat more rapid than the oxidation as

measured, but this is doubtless owing to the fact that the products of the reaction must diffuse from the spongy platinum into the solution before they can be detected by titration.

It seems evident, therefore, that in this reaction, as in the oxidation of formaldehyde, the platinum is capable of carrying oxygen into the solution, and it appears from fig. 3 that if the oxygen were supplied at sufficiently frequent intervals the reaction could be made to follow the curve *OE*, which represents the action of colloidal platinum in the oxidation of potassium iodide by hydrogen peroxide.

From these two reactions it may be concluded that when colloidal platinum is introduced into a mixture of hydrogen peroxide and an oxidizable substance the platinum takes up oxygen from the peroxide, thereby forming a more efficient oxidizing agent than the original hydrogen peroxide. The catalytic action of the platinum in this case, that is, its peroxidase action, therefore, depends upon its aptitude for forming unstable oxygen compounds when it is in contact with hydrogen peroxide.

Similar experiments with plant material will be reported on in a subsequent paper.

LABORATORY OF PLANT PHYSIOLOGY
    HARVARD UNIVERSITY